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Two Homogeneous Immunoassays for Pyridoxamine

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Protein conjugates of pyridoxal have been used to elicit anti-vitamin B₆ antibodies in rabbits. These antibodies have been incorporated into 2 homogeneous assays systems, a spin immunoassay, using a paramagnetic derivative of the vitamin as ligand, and a fluorescence enzyme immunoassay, using β -galactosidase conjugated to vitamin B₆ as the indicator molecule. These assay systems do not require fractionation steps, and could be the basis of analytical methodology for nutritional research or clinical diagnosis.

Key words: *pyridoxine – vitamin B₆ – immunoassay – enzyme immunoassay – electron spin resonance*

Introduction

Rapid analysis of vitamins in multiple samples is a promising application of immunochemistry. Such analytical techniques are needed for clinical or experimental studies of human or animal subjects or for analysis of food components. Immunoassays have been reported for water-soluble vitamins such as cobalamin (Endres et al., 1978) and pantothenic acid (Howe et al., 1979). However, the use of immunoassays is not limited to the water-soluble vitamins, for radioimmunoassays have been reported for vitamin A (Westfall and Wirtz, 1979) and vitamin D (Clemens et al., 1978). Several investigators elicited antibodies directed against conjugates of pyridoxal or pyridoxal phosphate (Cordoba et al. 1966, 1970; Ungar-Waron and Sela, 1966; Raso and Stollar, 1975; Thanassi and Cidlowski, 1980; Viceps-Madore et al., 1983). In this report, we describe antibodies which bind pyridoxamine, and the use of these in 2 immunoassay systems, one using electron spin resonance (Leute et al., 1972) and the other, fluorescent enzyme assay. Both assays are homogeneous, that is, conducted without a fractionation step to separate antibody-bound from unbound components.

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Materials and Methods

Protein-vitamin conjugates

Conjugates of keyhole limpet hemocyanin or bovine serum albumin were prepared by treating the proteins with pyridoxal and reducing the adducts with borohydride (Ungar-Waron and Sela, 1966). Conjugates prepared in this manner contained 10–20 mol of vitamin per 100,000 g of protein. Similarly, enzyme-vitamin conjugates were prepared for the enzyme immunoassay. β -Galactosidase (E.C. 3.2.1.21) from *E. coli* (2.5 mg, 320 U/mg, Sigma Chemical Company, St. Louis, MO) was dissolved in 1.25 ml 50 mM sodium bicarbonate, pH 8.0. An equal volume of 0.15 M pyridoxal HCl (Sigma) in the same buffer was added to the enzyme solution and incubated at 37°C for 4 h. The mixture was then cooled to 0°C and 2 ml of sodium borohydride solution (15 mg/ml) was added and the sample was dialyzed. The absorbance peak at 320 nm was used to calculate a conjugation ratio of 20 mol of vitamin/mol of protein.

Preparation of antibodies

Female New Zealand White rabbits were immunized with 2 intramuscular injections of 1 mg of pyridoxal-hemocyanin conjugate emulsified in complete Freund's adjuvant, at biweekly intervals. Boosts were administered subcutaneously at 2–4-week intervals, using immunogen emulsified in incomplete Freund's adjuvant. The titer and specificity of sera were assessed by immunodiffusion against pyridoxal conjugates and by radioimmunoassay, as described below.

Radioimmunoassay of antibodies

[³H]pyridoxine (1.5 Ci/mmol) and [³H]pyridoxamine (2.9 Ci/mmol) were obtained from Amersham Corporation, Arlington Heights, IL. The latter compound was prepared by hydrogenation of pyridoxine with tritium gas. Both compounds were purified by thin layer chromatography on silica. For assay, serum dilutions were incubated with 0.1 μ Ci of pyridoxine or 0.02 μ Ci of pyridoxamine for 15 min. For affinity determination, various concentrations of unlabeled ligand were included in the incubation mixture. The tubes were then chilled in an ice-water bath. One milligram of horse IgG was added to each tube (as carrier), and 2 ml of 50% saturated ammonium sulfate solution was added. After 15 min, the tubes were centrifuged (2000 \times g, 15 min), the supernatants removed, and the precipitates dissolved in 0.3 ml formic acid. The formic acid solutions were transferred to 7 ml scintillation vials, and counted in a Packard model 3255 scintillation counter, after addition of 5 ml scintillator (Aquasol-II, New England Nuclear, Boston, MA). The binding constant of the antibody for pyridoxamine was determined to be $K_a = 5 \times 10^6 \text{ M}^{-1}$ by the method of Scatchard (1949). Within experimental limits, the spin label (see below) was indistinguishable from free pyridoxamine in the binding assay.

Paramagnetic ligands for spin immunoassay

The tetramethylpiperidine-1-oxyl derivative of pyridoxamine was prepared as illustrated in Fig. 1. The details are as follows:

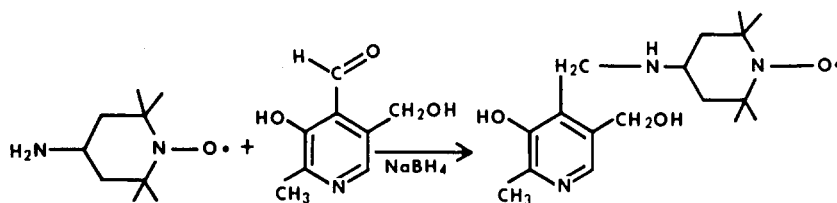


Fig. 1. The spin-labeled derivative of pyridoxamine was synthesized by reacting pyridoxal with 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl, followed by reduction with sodium borohydride.

Pyridoxylidene-4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl. A mixture of 2.03 g (0.01 mol) of pyridoxal HCl, 1.72 g (0.01 mol) of 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl, 1.4 g of potassium carbonate, 20 ml of H_2O , 20 ml of ethyl acetate and 100 mg of Adogen[®] 464 (Aldrich Chemical Co., Milwaukee, WI) were stirred overnight at room temperature. The organic layer was separated, and the aqueous layer was extracted with an equal volume of ethyl acetate. The combined extracts were washed with water, dried, and evaporated. The resulting crystals were recrystallized from methyl acetate-petroleum ether (m.p. 161–163°C). Elemental analysis – calculated for $C_{17}H_{26}N_3O_3$: C, 63.72; H, 8.18; N, 13.11. Found: C, 63.8; H, 8.2; N, 13.0.

4-N-(4'-(2',2',6',6'-tetramethylpiperidine-1-oxyl))-pyridoxamine. A solution of 0.15 g (0.004 mol) of sodium borohydride in 3 ml of water was added to a solution of 0.64 g of pyridoxylidene-4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl in 10 ml of 50% ethanol. After 30 min, 5 ml of acetone were added and the solvents were removed in vacuo. The product was recrystallized from methyl acetate-petroleum ether (m.p. 158–159°C). Elemental analysis – calculated for $C_{17}H_{28}N_3O_3$: C, 63.33; H, 8.75; N, 13.03. Found: C, 63.4; H, 8.69; N, 12.9.

Spin immunoassay

Assays were conducted by mixing 25 μ l of antibody, and 25 μ l of paramagnetic ligand. Spin label was present at a final concentration of 5×10^{-6} M. Samples were drawn into 50 μ l disposable micropipettes, one end of which was then sealed in a flame. Spectra were recorded on a Varian E-3 spectrometer (Varian Instruments, Palo Alto, CA) which was interfaced to a Nicolet Data System (Nicolet Instrument Corp., Madison, WI). All spectra were obtained at ambient temperature ($22^\circ C \pm 1^\circ C$).

Enzyme assays

Assays were performed at $37^\circ C$ in a final volume of 400 μ l, in 0.1 M sodium phosphate buffer, pH 7.3, containing 1 mM $MgCl_2$ and 110 mM 2-mercaptoethanol. The incubation mixture contained conjugated enzyme (10 μ l, 1 μ g/ml), and 4-methylumbelliferyl- β -galactopyranoside (Sigma Chemical Co.) was added to a final concentration of 20 mM to initiate the reaction. Incubations were terminated after 5 min by addition of 100 μ l 1 M sodium carbonate, and the fluorescence emission at 450 nm was determined with a Fluoricord spectrofluorometer (Baird-Atomic, Bed-

ford, MA), using excitation at 360 nm, and 8 nm entrance and exit slits. Colorimetric assays were used in some experiments and were performed similarly, but with *o*-nitrophenylgalactoside (Sigma Chemical Co.) as substrate (2.3 mM). The absorbance was read at 410 nm.

Results

Spin immunoassay

Three spectra were obtained at each pyridoxamine concentration using spectrum averaging of 100 scans per spectrum at a 3 s scan rate. The average peak-to-peak height of the low field line from the 3 spectra is plotted versus pyridoxamine concentration, giving the standard curve shown in Fig. 2. Control experiments indicated that the immobilization of spin label depended on the antibody activity and not on non-specific binding or viscosity effects. Incubation times from 1 min to several hours, at room temperature, produced similar results. The lower limit for detection of pyridoxamine under these conditions is about 5×10^{-7} M.

Fluorogenic enzyme immunoassay

Anti-pyridoxyl serum, but not normal serum, inhibits the conjugated enzyme, and the results in Table I indicate that the enzyme activity, in the presence of antibody, is sensitive to pyridoxamine. The degree of inhibition was insensitive to the duration of preincubation — periods of 30 min to 16 h produced comparable inhibition of

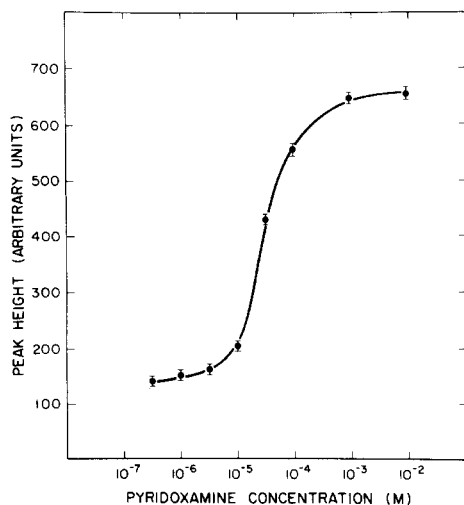


Fig. 2. Spin immunoassay of pyridoxamine. The equilibrium between free and antibody-bound spin label is shifted by the addition of unlabeled vitamin. The spectrum of the entire incubation mixture was recorded, and the peak height of the low-field line is plotted against the concentration of added pyridoxamine. Error bars indicate standard deviations.

TABLE I

DEPENDENCE OF β -GALACTOSIDASE ACTIVITY ON PYRIDOXAMINE AND PYRIDOXINE CONCENTRATION, IN THE PRESENCE OF ANTI-PYRIDOXYL ANTIBODIES

Pyridoxyl- β -galactosidase activity was determined colorimetrically with antiserum at a final dilution of 1:30.

Addition	Activity (arbitrary units)
None	100
Pyridoxamine	
3×10^{-6} M	100
3×10^{-5} M	153
3×10^{-4} M	220

activity. In some experiments, we found that goat anti-rabbit IgG (Miles Laboratories, Elkhart, IN) potentiates the inhibition by rabbit anti-pyridoxyl serum and reduces the background activity.

In an effort to reduce background activity, we determined the effect of goat anti-rabbit IgG (Miles Laboratories, Elkhart, IN). The results in Table II indicate that the second antibody does potentiate the inhibition by rabbit anti-pyridoxyl serum. There was no inhibition of activity by the goat antibody, in the absence of anti-pyridoxyl serum.

TABLE II

POTENTIATION OF INHIBITION OF PYRIDOXYL-GALACTOSIDASE ACTIVITY BY SECOND ANTIBODY

Enzyme activity was determined with rabbit antiserum at 1:512 and goat antibody at 70 μ g/ml. The enzyme concentration was 0.1 μ g/ml.

Additions	Activity (arbitrary units)
Buffer	100.0
Rabbit anti-pyridoxyl serum	62.0
Rabbit anti-pyridoxyl serum + goat anti-rabbit IgG	33.9

TABLE III

RELATIVE CONCENTRATION OF LIGAND NEEDED FOR 50% INHIBITION OF ANTIBODY BINDING

	Spin immunoassay	Enzyme immunoassay
Pyridoxamine	1	1
Pyridoxine	8.9	280
Pyridoxamine-5'-phosphate	28	35
Pyridoxic acid	$\gg 40$	> 1000

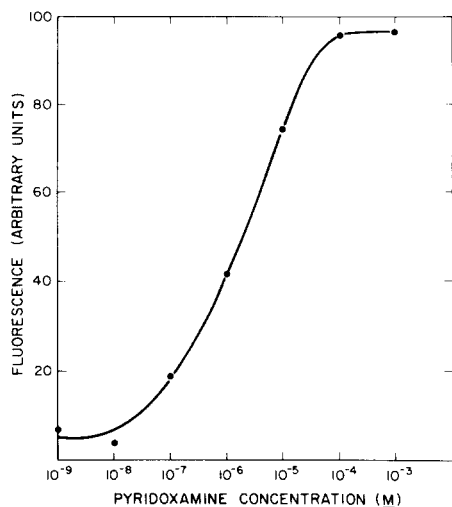


Fig. 3. Fluorogenic enzyme immunoassay for pyridoxamine. Enzyme activity was determined in the presence of rabbit anti-pyridoxyl serum (diluted 1:512) and goat anti-rabbit IgG (70 $\mu\text{g}/\text{ml}$). Fluorescence was monitored at 450 nm. The average coefficient of variation was 2%.

Fig. 3 illustrates the standard curve obtained for pyridoxamine, using rabbit anti-pyridoxyl and goat anti-rabbit IgG antibodies. The lower limit for the detection of pyridoxamine is approximately 10^{-8} M.

Assay specificity

Table III illustrates the specificity of the 2 immunoassays for pyridoxamine. Substituents in the 4-position strongly influence binding by antibody, and the addition of a 5'-phosphate ester also inhibits binding. The enzyme immunoassay is more specific for pyridoxamine than the spin immunoassay. This may reflect higher avidity binding of antibody to the enzyme conjugate resulting from divalent binding while only monovalent binding is possible for the spin label.

Discussion

This paper represents a first step in developing simple, homogeneous immunoassays for individual vitamers of B_6 . The results indicate that reduced Schiff's base conjugates of pyridoxal elicit antibodies selective for pyridoxamine. This is consistent with reports by previous investigators (Cordoba et al., 1966, 1970; Ungar-Waron and Sela, 1966), and is not surprising, in view of the immunogen used. In effect, the reduced Schiff's based adduct of pyridoxal and the ϵ -amino group of a lysyl residue is an alkylated derivative of pyridoxamine.

Spin immunoassay permits analysis of samples which are not optically clear, it does not require fractionation of the reaction mixture or the use of radioisotopes, and it is not likely to be sensitive to substances which interfere with other assays.

The most immediate improvement in the assay would be more sensitive detection of the magnetic absorption signal. We estimate that with the improved sensitivity of currently available spectrometers, the concentrations of spin-labeled hapten and antibody could be reduced to permit detection of pyridoxamine at about 2×10^{-9} M at a signal-to-noise ratio of 5:1. An additional improvement in sensitivity of another order of magnitude could be obtained by the use of a totally deuterated nitroxide moiety as the paramagnetic ligand.

Our results indicate that β -galactosidase, haptenated with pyridoxal, meets the essential requirements for a practical enzyme immunoassay. The enzyme is relatively stable, and its high turnover number permits sensitive assays. It should be possible to minimize the blank (enzyme activity in the absence of free vitamin), by removing poorly conjugated enzyme. In addition, samples which contain substances which quench the fluorescence could be assayed using a colorigenic substrate or a substrate with a different fluorochrome.

In summary, we have demonstrated that electron spin resonance and fluorescence spectroscopy can be adapted to the immunoassay of at least 1 vitamer of B_6 : pyridoxamine. The assays are homogeneous, requiring, in principle, minimal sample preparation. The detection limits of 0.1 ng for the enzyme immunoassay and 2.5 ng for the spin immunoassay compare favorably with recently described methods employing high performance liquid chromatography (Gregory, 1980; Gregory et al., 1981), which have a detection limit of about 1 ng. These assays, when extended to the quantitation of other vitamers of B_6 , should provide useful methodology for research in the areas of clinical and experimental nutrition, and for the analysis of foodstuffs.

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